IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	Anne R. Kubelik
Art Unit	:	1638
Applicants	:	Robert R. Schmidt, Philip Miller
Serial No.	:	09/070,844
Conf. No.	:	4873
Filed	:	May 1, 1998
For	:	Novel Polypeptides and Polynucleotides Relating to the Alpha- and Beta-Subunits of Glutamate Dehydrogenases and Methods of Use

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

THIRD DECLARATION OF PHILIP MILLER, Ph.D., UNDER 37 CFR §1.132

Sir:

I, Philip Miller, Ph.D., hereby declare and say as follows:

THAT, I am employed as a senior scientist at the Monsanto Corporation and a copy of my current *Curriculum Vitae* is attached hereto as Exhibit A;

THAT, I am an inventor of the subject matter described and claimed in the above-identified patent application;

THAT, by virtue of my education and professional occupation, including attending seminars and conferences, as well as keeping abreast of the scientific literature in my field, I am aware of the level of skill of the ordinary skilled artisan in the field of plant molecular biology;

THAT, I have studied the application Serial No. 09/070,844 and all office actions which have been issued during prosecution of this application as well as all responses which have been filed on the Applicants' behalf,

And, being thus duly qualified, do further declare as follows:

1. The subject application teaches one skilled in the art that plants transformed with glutamate dehydrogenase *a.k.a.* GDH (including NADP-GDH and NAD-GDH) will have altered (increased or decreased) nitrogen metabolism. It is acknowledged in the office action dated January 27, 2003, that the subject specification enables methods of increasing or decreasing nitrogen metabolism in plants by transforming the plants with a gene encoding a full-length NADP-GDH.

2. However, the office action maintains that the specification does not enable methods of using any GDH or fragments thereof to transform a plant so that the plant has increased or decreased nitrogen metabolism. The office action states that page 4 of the specification is directed to increasing nitrogen metabolism (*i.e.* using NADP-GDH-transformed plants) but that there is no teaching of a use for plants that have decreased nitrogen metabolism or for NAD-GDH-transformed plants.

3. I respectfully disagree with the assertions discussed above in paragraph 2. The specification discusses the benefits of decreasing nitrogen uptake to increase carbohydrate accumulation. For example, page 10, lines 7-9, of the specification states that "Expression of, for example, the β-homohexamers or GDH heterohexamers can be used to alter the rate of nitrogen assimilation, favoring accumulation of carbohydrates in fruits and other storage organs [emphasis added]." The benefits of this are readily apparent. For example, fruit plants modified in this manner can produce fruit that is bigger than the fruit of unmodified plants.

4. GDH catalyzes both directions of a two-way (reversible) reaction. In one direction is amination of 2-oxoglutarate to glutamate, which leads to increased nitrogen uptake. In the other direction is deamination of glutamate to yield 2-oxoglutarate, which leads to carbohydrate accumulation and decreased nitrogen uptake. This is mentioned on page 4 (for example) of the specification where it is stated that "[t]he alteration of nitrogen assimilation can have the effect of increasing nitrogen assimilation, which, as is well understood in the art, can affect the composition of the plant through an inverse effect on carbon metabolism [*e.g.* accumulation of carbohydrates]." Again, the two-way reaction that GDH catalyzes leads to nitrogen assimilation in one direction and carbohydrate accumulation (decreased nitrogen

metabolism) in the other direction. As illustrated by the above quote from page 10 of the specification and as would be readily understood by the ordinary skilled artisan, the application clearly teaches that both nitrogen assimilation and carbohydrate accumulation can be beneficial, depending on the situation and the desired result (*e.g.*, increased tolerance to ammonia and increased nitrogen uptake, or bigger fruit via increased carbohydrate accumulation and decreased nitrogen uptake). Thus, it is clear that the specification teaches benefits of using GDH to decrease nitrogen metabolism to favor carbohydrate accumulation, as well as the benefits of increasing ammonium assimilation.

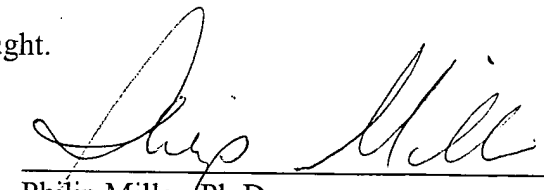
5. The office action states that it is not clear that NAD-GDH would work in a chloroplast and states that NADPH is the reductive nucleotide in the chloroplast (implying that NAD-dependant enzymes would not be expected to work in chloroplasts). However, there are many enzymes that are known to function within the chloroplast that use NAD as the reductive method. One does not have to look further than the nitrogen pathway (the subject of this application) to find another example of such an enzyme. For example, NADH-GOGAT can replace Fdx-GOGAT in the chloroplast. *See* page 369 of *Biochemistry and Molecular Biology of Plants*, eds. Buchanan, Gruissem, Jones, American Society of Plant Biologists, Rockville, MD, MD ISBN 0-943088-39-9 (copy attached). The ability of such an important NAD-dependent enzyme to function in chloroplasts should help to illustrate that reductive nucleotides in chloroplasts are more diverse than is characterized in the office action. A brief MEDLINE search revealed numerous other examples of NAD-dependent enzymes that function in chloroplasts. For example, Berkemeyer *et al.* relates to an NAD-dependant malate dehydrogenase from Arabidopsis chloroplasts, and Elortza *et al.* relates to a pea chloroplast NADH-plastoquinone oxide reductase. Dzelzkalns *et al.* note that chloroplasts contain genes with homology to NADH-ubiquinol oxidoreductase subunits. (Abstracts attached.) In addition, several references including "Photosynthesis: A Comprehensive Treatise" by A.S. Raghavendra mention that NADH can be transported to and from the mitochondria, peroxisome, and chloroplasts.

6. Furthermore, the GDH enzyme does not need to be targeted to the chloroplast. The production of glutamate and α-ketoglutarate are important to the invention. Changing the available pools of these important metabolic intermediates in the cytoplasm would also have distinct effects on the cell. As mentioned above, the reaction that GDH catalyzes is a two-way reaction. Thus, having the benefits of the teachings of the subject application, one of ordinary skill in the art could select a GDH (from a wide range of GDHs) and express it in plant cells (even in the cytoplasm) with the expectation that this would change the available pools of glutamate and α-ketoglutarate in the desired manner.

7. Regarding "fragments," the specification enables GDH enzymes, whether full-length or less-than-full-length, that are active in the reaction discussed above. One skilled in the art is aware that many enzymes (and other functional proteins) can be truncated to yield fragments that have the same activity (catalytic in the case of many enzymes) as that of the full-length proteins from which they are derived. Page 30, for example, of the specification describes this and also describes some methods for creating fragments of the enzymes described herein. Fragments of full-length GDH enzymes can be produced, their activity verified, and used according to the subject invention, with the expectation that an active fragment would catalyze this reaction, which is identified in the subject application as being important for achieving the desired result (*i.e.*, increased nitrogen accumulation or carbohydrate accumulation).

8. The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or of any patent issuing thereon.

9. Further declarant sayeth naught.

Signed: 

Philip Miller, Ph.D.

Date: 5·27·2003

Attachments: Exhibit A, *Curriculum Vitae*
Exhibit B, page 369 of *Biochemistry and Molecular Biology of Plants*
Exhibit C, abstracts from MEDLINE search:
Berkemeyer *et al.*, J. Biol. Chem. 1998, **43**:27927-27933.
Elortza *et al.*, Plant Cell Physiol. 1999, **40**(2):149-154.
Dzelzkalns *et al.*, Plant Physiol. 1994, **1106**(4):1435-1442.

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PROFESSIONAL ADDRESS

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PROFESSIONAL EMPLOYMENT

October 2002 to present	**Site Director, Monsanto Mystic CT. Research,** Lead of research Site including all operations, public/government affairs issues, and research programs. Program Director of cross-functional corn functional genomics efforts. Establishment and financial tracking of a multimillion dollar annual budget. Manage external relationships and review/align new technologies. Direction of broad research efforts on gene discovery, testing, metabolic and protein engineering of monocots and dicots. Utilizing broad range of enabling technologies to address elucidate biochemical and molecular regulation of pathways, abiotic stess responses, and feed and processing quality traits. Serve on Monsanto Scientific Advisory Teams. Lead of Ceres /Monsanto research collaboration. Technology Strategy Board member .
October 1999 to October 2002	**Program Director, Biotech Crop Yield Enhancement,** Pharmacia- Monsanto Subsidiary, St. Louis MO and Mystic , CT. Program Research Director of cross-functional crop yield biotechnology efforts. Establishment and financial tracking of a multimillion dollar annual budget. Manage external relationships and review/align new technologies. Direction of broad research efforts on metabolic and protein engineering of monocot and dicot crop and model systems. Utilizing broad range of enabling technologies to address elucidate biochemical and molecular regulation of plant metabolic pathways, abiotic stess responses, and plant architecture. Serve on Monsanto Scientific Advisory and Genomics Councils. Member of AG Technology Strategy Board.
July 1997 to October 1999	**Project Leader Corn/Wheat Yield Biotech,** Monsanto Life Sciences, St. Louis MO and Mystic , CT. Research direction and management of corn and wheat biotechnology efforts at two research locations consisting of seven Ph.D. senior scientists/postdoctoral associates and ten senior MS/BS scientists. Directing research, review technologies, prioritization and project development in carbohydrate/nitrogen metabolism interactions, stress tolerance, and plant hormone responses. Development and technical direction of yield enhancement genomics approaches.
September 1996 to July 1997	**Senior Biochemist,** Monsanto Life Sciences St. Louis, MO, Phytochemistry Program. Principle investigator for development of novel chemical hybridizing agents. Determination of mode of action of chemical gametocides and development of high-throughput *in vitro* and *in vivo* screens of gametocide/herbicide chemistries.
March, 1995 to September 1996	**Postdoctoral Associate,** Monsanto, St. Louis MO. Biochemistry and Molecular biology of plant nitrogen and carbon metabolism in wheat and maize..

June, 1994 to March 1995	**Postdoctoral Associate,** University of Florida, Plant Molecular and Cellular Biology Program, Gainesville, FL, Robert J. Ferl. Performing studies to analyze protein-protein and protein-DNA interactions involved in plant inducible gene regulation, cloning, characterization of key transcription factors. Characterization of 14-3-3 regulatory proteins in plants and influence of chromatin structure on gene expression. Development of Ligation Mediated PCR for plant *in vivo* footprinting.
January, 1990 to May, 1994	**Graduate Research Assistant,** University of Florida, Department of Microbiology and Cell Science, Gainesville, FL, Robert R. Schmidt. Conducted research on the biochemical and physical characterization of the gene, mRNAs, and proteins involved in the synthesis of the chloroplastic and mitochondrial glutamate dehydrogenases of *Chlorella* and *Arabidopsis* and the regulation of nitrogen metabolism. Regulation of amino acid biosynthesis plants and micro-organisms.
August, 1987 to December, 1989	**Graduate Research Assistant**, Appalachian State University, Department of Chemistry and Biology, Boone, NC. Performed cytogenetic analysis of eukaryotic organisms exposed to environmental chemical clastogens and radiation clastogens at the Savannah River Nuclear Site. Identified chemical and radioactive constituents and chromosomal anomalies. Faculty appointment teaching Organic Chemistry I/II
June, 1983 to August, 1984	**Assistant Breeder**, Akin Seed Company, St. Francisville, IL. Responsible for nursery maintenance, cross pollinating, and cataloging crosses in the development of new commercial inbred/hybrid maize lines. Nursery coordinator.

EDUCATION

1990-1994	Ph.D., Biochemistry and Molecular Biology Department of Microbiology and Cell Science University of Florida, Gainesville, Florida
1987-1989	M.S. program Department of Biology Appalachian State University, Boone, North Carolina
1980-1984	B.S., Major: Premed Department of Biology Bob Jones University, Greenville, South Carolina

PROFESSIONAL SOCIETIES AND COMMITTEES

Monsanto Operations Board 1998 - present
Monsanto Technology Alliance Team Reviewer 1995 -present
USDA/NSF Nitrogen metabolism and genomics program reviewer 1994-present
Chairman: Distinguished Contributors to Cell Science
American Society of Plant Physiologists
International Society for Plant Molecular Biology
American Association for the Advancement of Science
Pi Epsilon Phi Literary Society

HONORS AND AWARDS

Award of Excellence for Top Graduate Research 1995, University of Florida
President's Outstanding Student Award 1994, University of Florida
Sigma Xi Research Society
Phi Kappa Phi Honor Society
Gamma Sigma Delta Honor Society

Beta Beta Beta Biological Honor Society
National Honor Society
International Plant Biochemistry Award 1993

PUBLICATIONS

Lawit SJ, Miller PW, Dunn WI, Miriable JS, Schmidt RR (2003) Heterologous Expression of cDNAs Encoding *Chlorella sorokiniana* Glutamate Dehydrogenase Wild Type and Mutant Subunits in *Eschericia coli* and comparison of Kinetic and Thermal Stability Properties of their Homohexamers. Plant Mol Biol (in press).

Cheikh NC, Miller PW, Kishore GM (1999) Role of biotechnology in crop productivity in a changing environment. *In* Climate Change and Global Crop Productivity *eds*. Reddy and Hodges. CAB International Publishers.

Walkup JA, Loussaert DF, Weaver LM, Miller PW (1999) Chemical gametocide enhancement: Genesis root uptake and mobilization as a mechanism for improved wheat hybridization. (Submitted, MSL)

Miller PW, Dunn WI, Schmidt RR (1998) Alternative splicing of a precursor-mRNA encoded by the Chlorella sorokiniana NADP-specific glutamate dehydrogenase gene yields mRNAs for precursor proteins of isozyme subunits with different ammonium affinities. Plant Mol Biol 37: 243-263.

Daughtery CA, Rooney MJ, Miller PW, Ferl RJ (1996) Molecular organization and tissue-specific expression of an Arabidopsis 14-3-3 gene. Plant Cell 8: 1239-1248.

Bonner CA, Fischer RS, Schmidt RR, Miller PW, Jensen RA (1995) Distinctive enzymes of aromatic amino acid biosynthesis that are highly conserved in land plants are also present in the Chlorophyte alga Chlorella sorokiniana. Plant Cell Physiol 36: 1013-102.

Miller PW, Russell BL, Schmidt RR (1994) Transcription initiation site of a NADP-specific glutamate dehydrogenase gene and potential use of its promoter region to express foreign genes in ammonium-cultured *Chlorella sorokiniana* cells. J Appl Phycol 6:211-223.

Cock JM, Kim KD, Miller PW, Hutson RG, Schmidt RR (1991) A nuclear gene with many introns encoding ammonium-inducible chloroplastic NADP-specific glutamate dehydrogenases in *Chlorella sorokiniana.* Plant Mol Biol 17:1023-1044.

TECHNICAL BULLETINS AND ABSTRACTS

Miller PW, Dunn WD, Schmidt RR (1994) Preparative nondenaturing gel electrophoresis to purify NADP-specific glutamate dehydrogenase from *Chlorella.* Bio-Rad Technical Bulletin US/EG 1897

Miller PW, Russell BL, Schmidt RR (1993) Potential use of an algal ammonium-inducible promoter to express foreign genes in *Chlorella.* J Phycol 29:16 abstract No. 84.

PATENTS

Miller PW, Schmidt RR. Novel polypeptides and polynuclueotides relating to the α- and β-subunits of Glutamate Dehydrogenase and methods of use. Docket # UF155. Patent issued 1998

Eight patents Pending

GRANT AWARDS

Brain proteins in plants: The Arabidopsis GF14 gene family. USDA Project 9303095, FLA-HOS-03281. Investigator: Robert J Ferl Coinvestigators: Philip Miller; Paul Sehnke

DOE-Oakridge Universities Research Grant

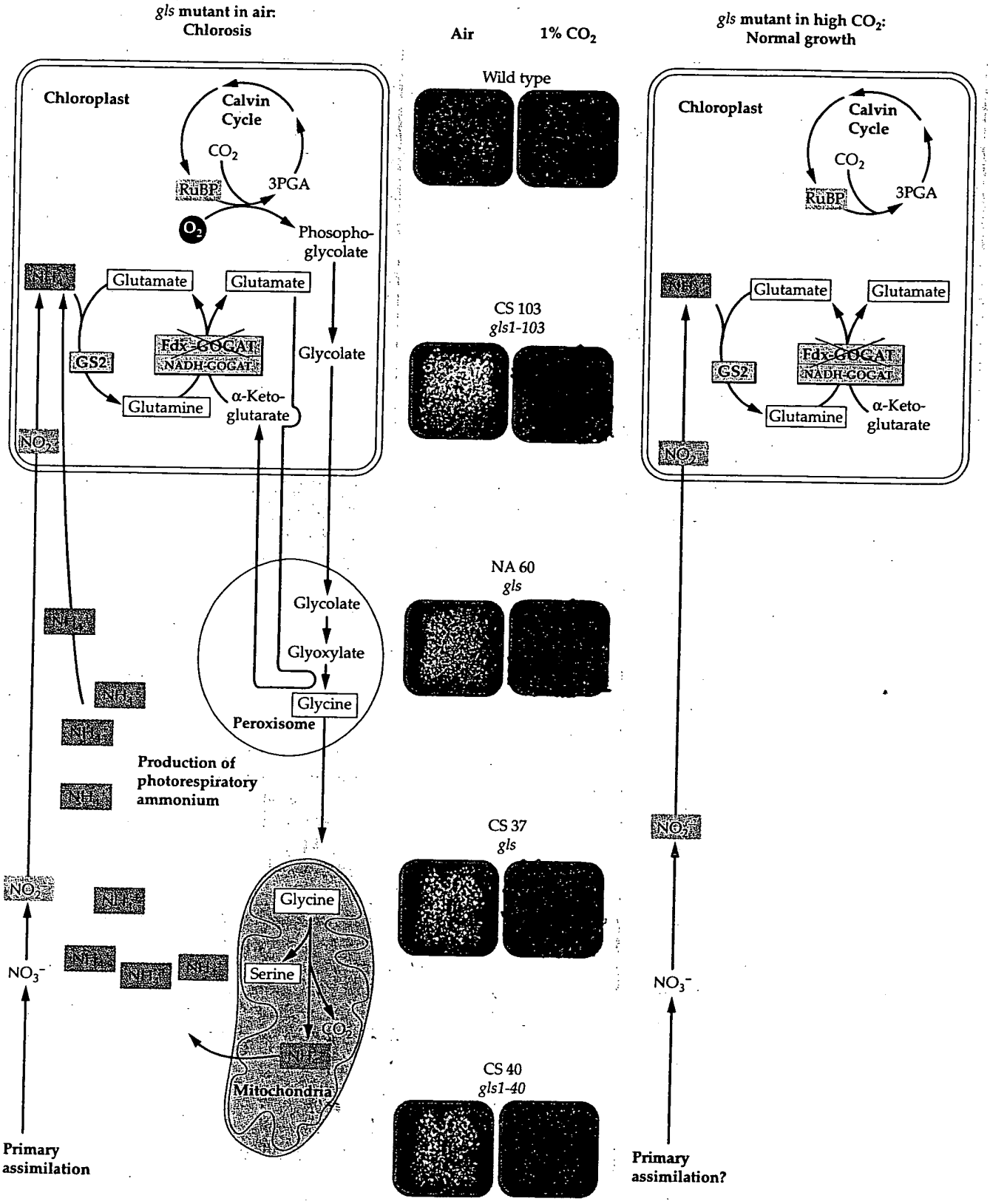

Figure 8.11
Proposed role of Fdx-GOGAT in primary nitrogen assimilation and in assimilation of ammonium produced as a by-product of photorespiration. *gls* (Fdx-GOGAT) mutants of *Arabidopsis* demonstrate a chlorotic phenotype when grown in air (approximately 0.03% CO_2) but can tolerate growth in a 1% CO_2 atmosphere that suppresses photorespiration.

1: J Biol Chem 1998 Oct 23;273(43):27927-33

A novel, non-redox-regulated NAD-dependent malate dehydrogenase from chloroplasts of Arabidopsis thaliana L.

Berkemeyer M, Scheibe R, Ocheretina O.

Pflanzenphysiologie, Fachbereich Biologie/Chemie, Universitat Osnabruck, D-49069 Osnabruck, Germany.

We report a novel plastidic NAD-dependent malate dehydrogenase (EC 1. 1.1.37), which is not redox-regulated in contrast to its NADP-specific counterpart (EC 1.1.1.82). Analysis of isoenzyme patterns revealed a single NAD-MDH associated with highly purified chloroplasts isolated from Arabidopsis and spinach. A cDNA clone encoding the novel enzyme was found in the Arabidopsis EST data base by sorting all putative clones for NAD-dependent malate dehydrogenase. A derived amino acid sequence is very similar to mitochondrial and peroxisomal NAD-MDHs within the region coding for the mature protein but possesses a 80-amino acid long N-terminal domain with typical characteristics of a chloroplast transit peptide. In vitro synthesized labeled precursor protein was imported into the stroma of spinach chloroplasts and processed to a mature enzyme subunit of 34 kDa. Expressed in Escherichia coli, the recombinant enzyme exhibited the same distinctive isoelectric point of 5.35 as the original enzyme from Arabidopsis chloroplasts. Northern analysis revealed that the protein is expressed in both autotrophic and heterotrophic tissues. The findings reported here indicate that the "malate valve" operates not only in the illuminated chloroplasts but also in dark chloroplasts and in heterotrophic plastids and is therefore a general mechanism to maintain the optimal ratio between ATP and reducing equivalents in plastids.

PMID: 9774405 [PubMed - indexed for MEDLINE]

1: Plant Cell Physiol 1999 Feb;40(2):149-54

Chloroplast NADH dehydrogenase from Pisum sativum: characterization of its activity and cloning of ndhK gene.

Elortza F, Asturias JA, Arizmendi JM.

Biokimika eta Biologia Molekularreko Saila, Euskal Herriko Unibertsitatea, Bilbao, Spain.

The pea chloroplast ndhK gene coding for a component of a NADH-plastoquinone oxidoreductase has been cloned and sequenced. This gene codes for a polypeptide of 227 amino acids and a predicted molecular mass of 25,495 Da which belongs to the family of the 20 kDa PSST subunit of the bovine mitochondrial complex I. A fragment of this gene has been overexpressed in Escherichia coli, and antibodies against the expressed polypeptide recognize a protein of the predicted molecular mass from pea thylakoid membranes. This polypeptide is a component of a protein complex with NADH dehydrogenase activity and is not associated with ferredoxin-NADP+ reductase.

PMID: 10202810 [PubMed - indexed for MEDLINE]

1: Plant Physiol 1994 Dec;106(4):1435-42 Related Articles, Links

Deletion of the structural gene for the NADH-dehydrogenase subunit 4 of Synechocystis 6803 alters respiratory properties.

Dzelzkalns VA, Obinger C, Regelsberger G, Niederhauser H, Kamensek M, Peschek GA, Bogorad L.

Department of Biology, Case Western Reserve University, Cleveland, Ohio 44106.

Chloroplasts and cyanobacteria contain genes encoding polypeptides homologous to some subunits of the mitochondrial respiratory NADH-ubiquinol oxidoreductase complex (NADH dehydrogenase). Nothing is known of the role of the NADH dehydrogenase complex in photosynthesis, respiration, or other functions in chloroplasts, and little is known about the specific roles of the perhaps 42 subunits of this complex in the mitochondrion. Inactivation of a gene for subunit 4 (ndhD-2, ndh4) of this complex in the cyanobacterium Synechocystis 6803 has no effect on photosynthesis, judging from the rate of photoautotrophic growth of mutant cells, but the mutant's respiratory rate is about 6 times greater than that of wild-type cells. Respiratory electron transport activity in cyanobacteria is associated both with photosynthetic thylakoid membranes and with the outer cytoplasmic membrane of the cell. Cytoplasmic membranes of mutant cells have much greater NADH-dependent cytochrome reductase activity than preparations from wild-type cells; this activity remains at wild-type levels in isolated thylakoid membranes. It is suggested that the 56.6-kD product of ndhD-2 is not essential for the activity of a cytoplasmic membrane-bound NADH dehydrogenase but that it regulates the rate of electron flow through the complex, establishing a link between this ndh gene and respiration. The activity of the molecularly distinct thylakoid-bound NADH dehydrogenase is apparently unaffected by the loss of ndhD-2.

PMID: 7846157 [PubMed - indexed for MEDLINE]

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